Conformational Change of *Arabidopsis thaliana* Thioredoxin Reductase after Binding of Pyridine Nucleotide and Thioredoxin

Henrikas Nivinskas[a], Jean-Pierre Jacquot[b], and Narimantas Čėnas[a,*]

[a] Institute of Biochemistry, Mokslininkų 12, Vilnius 2600, Lithuania.
Fax: 370–2-729196. E-mail: ncenas@bchi.lt

[b] Laboratoire de Biologie Forestiere, Universite de Nancy 1, 54506 Vandoeuvre Cedex, France

* Author for correspondence and reprint requests

Z. Naturforsch. **56 c,** 188–192 (2001); received November 30, 2000/January 16, 2001

Thioredoxin, Thioredoxin Reductase, *Arabidopsis thaliana*

We have found that the binding of $NADP^+$ (K_d = 0.86±0.11 μM) enhanced the FAD fluorescence of *Arabidopsis thaliana* NADPH:thioredoxin reductase (TR, EC 1.6.4.5) by 2 times, whereas the binding of 3-aminopyridine adenine dinucleotide phosphate ($AADP^+$) (K_d < 0.1 μM) quenched the fluorescence by 20%. Thioredoxin (TRX) also enhanced the FAD fluorescence by 35%. The K_d of TR-$NADP^+$ and TR-$AADP^+$ complexes did not change in the presence of 45 μM TRX. Our findings imply that the binding of $NADP^+$ and $AADP^+$ at the NADP(H)-binding site of *A. thaliana* TR, and/or the binding of TRX in the vicinity of the catalytic disulfide increase the content of fluorescent FR conformer (NADP(H)-binding site adjacent to flavin). The different effects of $NADP^+$ and $AADP^+$ on FAD fluorescence intensity may be explained by the superposition of two opposite factors: i) increased content of fluorescent FR conformer upon binding of $NADP^+$ or $AADP^+$; ii) quenching of FAD fluorescence by electron-donating 3-aminopyridinium ring of $AADP^+$.

Introduction

Thioredoxin (TRX), a disulfide-reducing redox protein, and flavoenzyme NADPH: thioredoxin reductase (TR, EC 1.6.4.5) form a ubiquitous redox system in both prokaryotic and eukaryotic organisms, which participates in cell proliferation, light-mediated enzyme regulation in oxygenic photosynthesis and germination of plants, and performs antioxidant functions (Holmgren, 1985; Lunn and Pigiet, 1987; Fernando *et al.,*1992; Jacquot *et al.,* 1997; Powis *et al*., 1998).

The best characterized TR from *Escherichia coli* is a homodimer and contains one FAD and one redox active disulfide (Cys135, Cys138) per 33 kDa subunit (Williams, 1995). The catalysis of *E. coli* TR shares analogous steps with other NAD(P)H: disulfide reductases, and involves the transfer of redox equivalents from NADPH to FAD, from reduced FAD to catalytic disulfide of TR, and then from the newly formed enzyme dithiol to the disulfide of TRX (Williams, 1995). However, the crystal structure of TR markedly differs from other NADPH:disulfide reductases. NADP(H) and disulfide substrates of glutathione and trypanothione reductases bind at the vicinity of the FAD and catalytic disulfide, respectively, their binding sites being separated by the isoalloxazin ring of FAD (Williams, 1995), whereas in TR, NADP(H) binds to the domain containing the catalytic disulfide, the nicotinamide ring being located 17 Å away from the flavin ring (Waksman *et al.,* 1994; Williams, 1995). The access of NADP(H) to FAD (or access of TRX to catalytic disulfide) probably requires the rotation of the NADP(H)-catalytic disulfide domain with respect to the FAD domain. It has been proposed that during catalysis, *E. coli* TR exists in an equilibrium between two conformers, nonfluorescent FO (catalytic disulfide adjacent to FAD), and fluorescent FR (NADP(H) binding site adjacent to FAD) (Waksman *et al.,* 1994; Williams, 1995; Wang *et al.,* 1996; Mulrooney and Williams, 1997) (Fig. 1). Kinetic studies of covalent mixed disulfide TR-TRX complexes (Wang *et al.,* 1996), and the data of fluorescence quenching studies of Cys138Ser mutant of *E. coli* TR

Abbreviations: TR, NADPH:thioredoxin reductase; TRX, thioredoxin; $AADP^+$, 3-amino-pyridine adenine dinucleotide phosphate.

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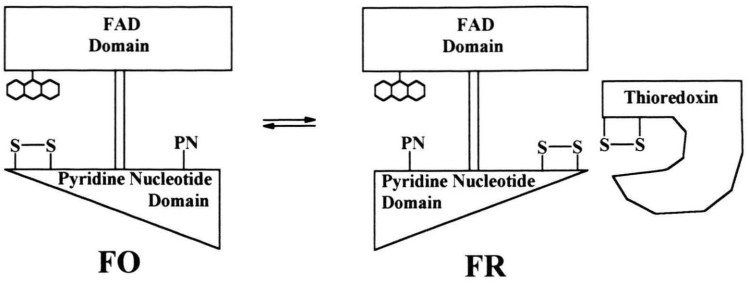

Fig. 1. Diagram of the two forms of thioredoxin reductase. The FAD and pyridine nucleotide domains are indicated. The tricyclic ring represents the FAD, and PN indicates bound pyridine nucleotide.

(Mulrooney and Williams, 1997), are in line with this assumption.

NADPH:thioredoxin reductase from *Arabidopsis thaliana* is a plant-type cytosolic enzyme possessing 45% sequence homology and analogous three dimensional structure to *E. coli* TR (Dai *et al.,* 1996). Recent steady-state kinetics studies also point out to an analogous mechanism of catalysis of *A. thaliana* and *E. coli* thioredoxin reductases (Bironaitė *et al.,* 1998; Miškinienė *et al.,* 1998). The FAD fluorescence studies of *A. thaliana* TR, performed in the present study, provide good evidence for the transition between the FO and FR conformational states of this enzyme.

Materials and Methods

A. thaliana thioredoxin reductase and thioredoxin, and *Chlamydomonas reinhardtii* tioredoxin were prepared as described previuosly (Jacquot *et al.,* 1994; Stein *et al.,* 1995). Concentrations of TR and TRX were determined spectrophotometrically, using ε_{454} = 11.3 $mM^{-1}cm^{-1}$ and ε_{280} = 10.9 $mM^{-1}cm^{-1}$ (Jacquot *et al.,* 1994; Stein *et al.,* 1995), respectively. NADPH, $NADP^+$, $AADP^+$, 2′,5′-ADP and other reagents were obtained from Sigma. All experiments were performed in 0.1 M K-phosphate buffer solution (pH 7.0) containing 1 mM EDTA, at 25 °C. The activity of TR determined using the TRX-mediated reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (11,12), was equal to 7 mol NADPH oxidized per mole FAD per 1 s (concentation of NADPH, 40 μM, concentration of TRX, 20 μM, concentration of DTNB, 300 μM). The absorbance spectra have been recorded using a Hitachi-557 spectrophotometer. The fluorescence spectra of FAD of TR (470–670 nm) were recorded using a Hitachi MPF-4 spectrophotometer (excitation wavelength 455 nm, enzyme concentration 2.0–5.0 μM). The dissociation constants (K_d) of TR complexes with $NADP^+$ and its analogs were calculated from the fluorescence intensity changes (ΔI) by formulae taken from Tuls *et al.,* (1987): $\Delta I/\Delta I_{max} = (B - (B^2 - 4[E][L])^{0.5})/2[E]$, where ΔI_{max} is the maximal fluorescence intensity change at fully saturating ligand concentration, [E] is enzyme concentration, [L] is the ligand concentration, and B = [E] + [L] + K_d.

Results and Discussion

On analogy with TR from *E. coli* (Wang *et al.,* 1996; Mulrooney and Williams, 1997), the addition of stoichiometric amount of 3-amino-pyridine adenine dinucleotide phosphate ($AADP^+$) changed the absorbance spectra of FAD of *A. thaliana* TR (Fig. 2), whereas the addition of 10–100 μM $NADP^+$ or TRX (30–40 μM) caused much less expressed changes (data not shown).

As observed for *E. coli* TR (Mulrooney and Williams, 1997), FAD of *A. thaliana* enzyme was fluorescent with λ_{max} = 518 nm, possessing approx. 25% of the fluorescence intensity of free FAD. The addition of $AADP^+$ quenched the FAD fluorescence (Fig. 3A). The maximal extent of quenching (20%) was markedly lower compared to the one reported for *E. coli* TR, 75% (Mulrooney and Williams, 1997). In contrast, $NADP^+$ *enhanced* the FAD fluorescence (Fig. 3A), the maximal increase in fluorescence being equal to 100%, and the K_d of TR-$NADP^+$ complex being equal to 0.86±0.11 μM. This was in a marked contrast to *E. coli* TR, where $NADP^+$ does not affect the FAD fluorescence (Williams, C. H., Jr., personal communication). In view of the relatively small absorbance and fluorescence changes and high affinity of $AADP^+$ to TR, we were unable to determine the

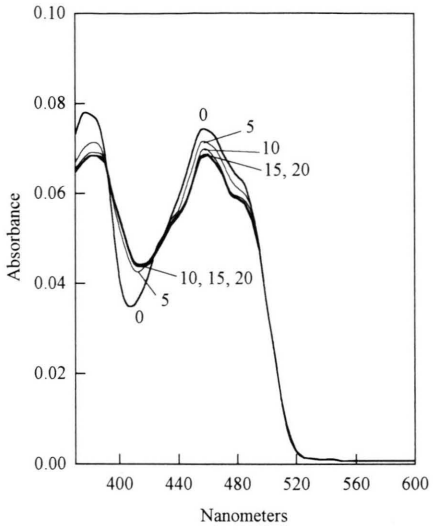

Fig. 2. Absorbance spectra of thioredoxin reductase (6.7 μM) in the absence and in the presence of various concentrations of AADP$^+$. The numbers indicate the concentration of AADP (μM).

K_d of TR-AADP$^+$ complex directly. On the other hand, 2,5′-ADP also enhances the FAD fluorescence by ΔI_{max} = 28% (K_d = 23.8±4.5 μM). The presence of 2′,5′-ADP in the medium leads to the increase in K_d of TR-NADP$^+$ and TR-AADP$^+$ complexes (Fig. 3B). Obviously, the above compounds compete for an identical binding site. The extrapolation of the data of Fig. 3B to zero concentration of 2′,5′-ADP gives the approximate value of K_d of TR-AADP$^+$ complex, 0.1 μM. Thus, the affinity of *A. thaliana* TR to AADP$^+$ is around 100 times higher than the affinity of *E. coli* TR (K_d = 8.0 μM (Mulrooney and Williams, 1997).

In accordance to our previous data (Miškinienė *et al.*, 1998), TRX enhanced the FAD fluorescence of TR (Fig. 4A). The sigmoidal dependence of fluorescence increase on TRX concentration points to possible cooperativity in TRX binding. The maximal fluorescence increase has been reached after addition of 15–25 μM *A. thaliana* TRX, or at higher concentrations of *Cl. reinhardtii* TRX, 30–40 μM (Fig. 4A). This was in line with the K_m values of *A. thaliana* TRX (K_m = 1.2 μM) and *Cl. reinhardtii* TRX (K_m = 2.6 μM) in the steady-state reactions of TR (Bironaitė *et al.*, 1998). However, since *Cl. reinhardtii* TRX enhanced the FAD fluorescence by a larger extent (35%) as compared to *A. thaliana* TRX (20%) (Fig. 4A), all the subsequent experiments were performed in the presence of *Cl. reinhardtii* TRX. The subsequent addition of NADP$^+$ further increased the fluorescence, whereas the addition of AADP$^+$ quenched it (Fig. 4B). However, the presence of 45 μM *Cl. reinhardtii* TRX did not alter the affinity of NADP$^+$ and AADP$^+$ to TR, since the K_d of corresponding complexes were equal to 0.59±0.11 μM and 0.07±0.013 μM.

Our findings imply that the binding of TRX in the vicinity of the catalytic disulfide of TR may cause conformational change increasing the concentration of the fluorescent TR conformer (NAD(P)H-binding site adjacent to flavin) (Fig. 1,3A). This is understandable from purely sterical reasons. Interestingly, the binding of

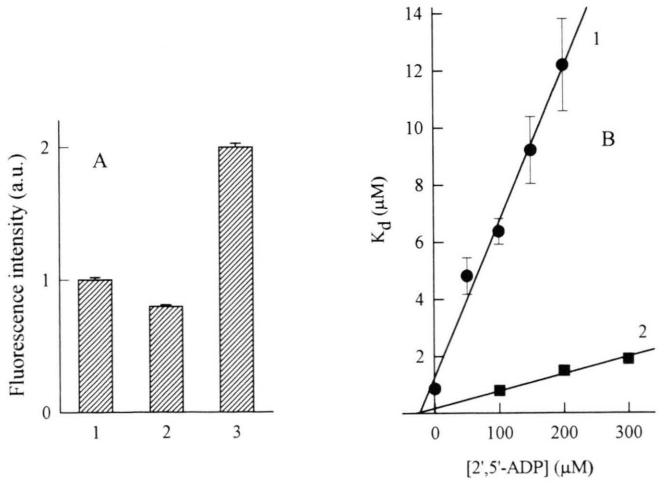

Fig. 3. **A)** Relative fluorescence intensity of the FAD of thioredoxin reductase (3.0 μM) in control experiment (1), in the presence of 10 μM AADP$^+$ (2), and in the presence of 15 μM NADP$^+$ (3). Excitation wavelength, 455 nm, emission wavelength, 518 nm. **B)** The dependence of K_d of TR-NADP$^+$ (1) and TR-AADP$^+$ complexes (2) on the concentration of 2′,5′-ADP.

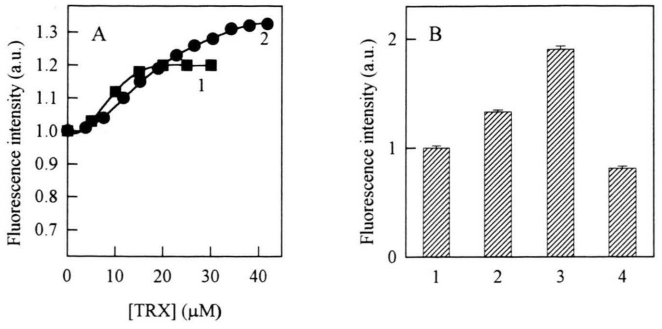

Fig. 4. **A)** Increase in fluorescence intensity of the FAD of thioredoxin reductase (2.5 μM) in the presence of various concentrations of *A. thaliana* (1) and *Cl. reihardtii* TRX (2). **B)** Relative fluorescence intensity of the FAD of TR (3.0 μM) in control experiment (1), in the presence of 45 μM *Cl. reihardtii* TRX (2), in the presence of 20 μM $NADP^+$ + 45 μM TRX (3), in the presence of 15 μM $AADP^+$ + 45 μM TRX (4). Excitation wavelength, 455 nm, emission wavelength, 518 nm.

$NADP^+$ or 2′,5′-ADP to *A. thaliana* TR also enhanced the FAD fluorescence (Fig. 2), thus pointing to a possibility that the occupation of NADP(H) binding site by $NADP^+$, or even adenosine ribose 2′-phosphate binding site by 2,5′-ADP, also shifts the equilibrium in favour of TR conformer. This observation gives some information on the initial steps of reductive half-reaction of TR, namely, that the binding of NADPH to its binding site may induce the conformational transition of TR, which adopts the geometry appropriate for a subsequent hydride transfer from NADPH to FAD. Previously, the flavin movement from the buried to exposed conformation prior to the reaction with NADPH has been detected for p-hydroxybenzoate hydroxylase (Palfey *et al.*, 1999). The different effects of $NADP^+$ and $AADP^+$ on FAD fluorescence intensity may be explained by the superposition of two opposite factors: i) increased content of fluorescent FR conformer upon binding of $NADP^+$ or $AADP^+$; ii) quenching of FAD fluorescence by electron-donating 3-aminopyridinium ring of $AADP^+$.

Finally, the differences between *E. coli* (Mulrooney and Williams, 1997) *A. thaliana* TR with respect to the effects of pyridine nucleotides on the FAD fluorescence are quantitative but not qualititative. In *E. coli* TR, $AADP^+$ significantly quenches FAD fluorescence, and $NADP^+$ does not affect it, whereas in *A. thaliana* TR, $AADP^+$ quenches FAD fluorescence insignificantly, and $NADP^+$ enhances it. This might be attributed to some differences in the relative positions of *E. coli* and *A. thaliana* FAD- and NADP(H)-binding domains, since the NADP(H) domain of *A. thaliana* TR must be rotated by 8° to superimpose on the corresponding domain of the *E. coli* enzyme (Waksman *et al.*, 1994; Dai *et al.*, 1996). Nevertheless, our data provide simple and straightforward evidence for the conformational transition between FO and FR states, that is shared by TR from both sources.

Acknowledgements

This work was supported in part by NATO Linkage grant HTECH.LG972012.

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